

**Preliminary Amendment**

Page 7 of 7

Applicant(s): Boldogh et al.

Serial No.: 09/641,802

Confirmation No.: unknown

Filed: August 17, 2000

For: USE OF COLOSTRININ, CONSTITUENT PEPTIDES THEREOF, AND ANALOGS THEREOF TO PROMOTE NEURAL CELL DIFFERENTIATION

**REMARKS**

These amendments simply correct typographical errors in the documents referenced or more accurately describe the documents, and add no new matter to the specification.

The amendment made on pages 5, 15, and 16 were to remove a reference to "et al." on an article with one author.

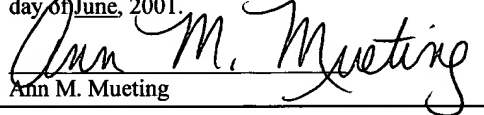
The amendment made to the paragraph spanning pages 6 through 8 was made to provide the current publication number of the PCT document.

The amendment to page 11 corrects a typographical error.

The Examiner is invited to contact Applicants' Representatives, at the below-listed telephone number, if it is believed that prosecution of this application may be assisted thereby.


**CERTIFICATE UNDER 37 C.F.R. 1.8:**

The undersigned hereby certifies that this paper is being deposited in the United States Postal Service, as first class mail, in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231, on this 12 day of June, 2001.

  
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Appendix A  
Specification Amendments with Notations to Indicate Changes Made

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Brackets are used to signify deletions, and underlining is used to signify additions herein.  
The changes are also shaded in gray.

Page 5, line 21 to line 30

The differentiation process of cells in the nervous system is regulated by the action of differentiation and growth factors including NGF. For example, NGF binding to its receptor tyrosine kinase, TrkA, initiates various molecular interactions including tyrosine phosphorylation of proteins and the action of the Ras/Raf/MEK/MAPK pathway (Chao[et al.], Cell, 68, 995-997 (1992); and Marshall et al., Cell, 80, 179-185 (1995)). NGF induces the production of reactive nitric oxide (NO), and NO is required for NGF-induced cytoskeleton and differentiation (Peunova et al., Nature, 375, 68-73 (1995)), suggesting that free radical molecules may exert a regulatory role in certain types of cellular differentiation.

**Page 6, line 26 to page 8, line 3**

Colostrinin has been found to include a number of peptides ranging from 3 amino acids to 22 amino acids or more. These can be obtained by various known techniques, including isolation and purification involving electrophoresis and synthetic techniques. The specific method of obtaining colostrinin and SEQ ID NO:31 is described in International Publication No. WO-A-98/14473. Using HPLC and Edelman Degradation, over 30 constituent peptides of colostrinin have been identified, which can be classified into several groups: (A) those of unknown precursor; (B) those having a  $\beta$ -casein homologue precursor; (C) those having a  $\beta$ -casein precursor; and (D) those having an annexin precursor. These peptides are described in International Patent [Application PCT/GB00/02128] Publication No. WO/00/75173, filed June 2, 2000, claiming priority to June 2, 1999, and can be synthesized according to the general method described in the Examples Section. These peptides (i.e., constituent peptides of colostrinin), which can be derived from colostrinin or chemically synthesized, include: MQPPPLP (SEQ ID NO:1); LQTPQPLLQVMMEPQGD (SEQ ID NO:2); DQPPDVEKPDLPFQVQS (SEQ ID NO:3); LFFFLPVNVLP (SEQ ID NO:4); DLEMPVLPVEPFPPV (SEQ ID NO:5); MPQNFYKLPQM (SEQ ID NO:6); VLEMKFPPPPQETVT (SEQ ID NO:7); LKPFPKLKVEVFPFP (SEQ ID NO:8); VVMEV (SEQ ID NO:9); SEQP (SEQ ID NO:10); DKE (SEQ ID NO:11); FPPPK (SEQ ID NO:12); DSQPPV (SEQ ID NO:13); DPPPPQS (SEQ ID NO:14); SEEMP (SEQ ID NO:15); KYKLQPE (SEQ ID NO:16); VLPPNVG (SEQ ID NO:17); VYPFTGPIP (SEQ ID NO:18); SLPQNILPL (SEQ ID NO:19); TQTPVVVPPF (SEQ ID NO:20); LQPEIMGVPKVKETMVPK (SEQ ID NO:21); HKEMPFPKYPVEPFTESQ (SEQ ID NO:22); SLTLTDVEKLHLPLPLVQ (SEQ ID NO:23); SWMHQPP (SEQ ID NO:24); QPLPPTVMFP (SEQ ID NO:25); PQSVLS (SEQ ID NO:26); LSQPKVLPVPQKAVPQRDMPIQ (SEQ ID NO:27); AFLLYQE (SEQ ID NO:28); RGPFPILV (SEQ ID NO:29); ATFNRYQDDHGEEILKSL (SEQ ID NO:30); VESYVPLFP (SEQ ID NO:31); FLLYQEPVLGPVR (SEQ ID NO:32); LNF (SEQ ID NO:33); and

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MHQPPQPLPPTVMFP (SEQ ID NO:34). These can be classified as follows: (A) those of unknown precursor include SEQ ID NOs:2, 6, 7, 8, 10, 11, 14, and 33; (B) those having a  $\beta$ -casein homologue precursor include SEQ ID NOs:1, 3, 4, 5, 9, 12, 13, 15, 16, 17, and 31; (C) those having a  $\beta$ -casein precursor include SEQ ID NOs:18 (casein amino acids 74-83), 19 (casein amino acids 84-92), 20 (casein amino acids 93-102), 21 (casein amino acids 103-120), 22 (casein amino acids 121-138), 23 (casein amino acids 139-156), 24 (casein amino acids 157-163), 25 (casein amino acids 164-173), 26 (casein amino acids 174-179), 27 (casein amino acids 180-201), 28 (casein amino acids 202-208), 29 (casein amino acids 214-222), 32 (casein amino acids 203-214), and 34 (casein amino acids 159-173); and (D) those having an annexin precursor include SEQ ID NO:30 (annexin amino acids 203-220).

**Page 11, line 4 to page 11, line 19**

For instance, peptide/carrier molecule conjugates may be prepared by treating a mixture of peptides and carrier molecules with a coupling agent, such as a carbodiimide. The coupling agent may activate a carboxyl group on either the peptide or the carrier molecule so that the carboxyl group can react with a nucleophile (e.g., an amino or hydroxyl group) on the other member of the peptide/carrier molecule, resulting in the covalent linkage of the peptide and the carrier molecule. For example, conjugates of a peptide coupled to ovalbumin may be prepared by dissolving equal amounts of lyophilized peptide and ovalbumin in a small volume of water. In a second tube, 1-ethyl-3-(3-dimethylamino-propyl)-[carbodiimide] carbodiimide hydrochloride (EDC; ten times the amount of peptide) is dissolved in a small amount of water. The EDC solution was added to the peptide/ovalbumin mixture and allowed to react for a number of hours. The mixture may then dialyzed (e.g., into phosphate buffered saline) to obtain a purified solution of peptide/ovalbumin conjugate. Peptide/carrier molecule conjugates prepared by this method typically contain about 4 to 5 peptides per ovalbumin molecule.

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Page 15, line 30 to page 16, line 14

**Methods:** To evaluate the effect of colostrum, colostrinin and its component peptides on cell differentiation  $3 \times 10^4$  logarithmically replicating (70% confluence) PC12 cells were seeded in 24 x well plates and cells were allowed to adhere and grow for 24 hours. Serum containing media were aspirated and replaced for serum-free RPMI containing appropriate amount of antibiotics. In four parallel, increasing concentrations (0.1, 1, 10 and 100  $\mu\text{g}$  per ml) of colostrum, colostrinin and its component peptides were added directly into the media and incubated at 37°C. As a positive control, nerve growth factor (NGF) 7S (Gibco-BRL) was used at 100 ng per ml concentration (Chao[et al.], *Cell*, 68, 995-997 (1992); and Marshall et al., *Cell*, 80, 179-185 (1995)). Phorbol 12-myristate 13-acetate (TPA: 10 ng per ml) was used as a negative control. Eight hours later the media were changed and RPMI-1640 was added containing 1% or 10% fetal bovine serum. The cultures were microscopically investigated 2<sup>nd</sup>, 4<sup>th</sup> and 6<sup>th</sup> days after treatment. Six days after treatment cells were fixed with paraformaldehyde (4%) and stained with 0.01% crystal violet solution. Excess of dyes were removed by ethanol washing. The final evaluation took place using a microscopy (*Axiophot2* Zeiss Inc., Germany).

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Page 16, line 16 to line 28

RESULTS

Control non-treated cells demonstrated the usual rounded morphology, continued to replicate and reach 70% to 80% confluency during 7 days experimental period. The mock-treated control and TPA exposed cells showed a low background level of differentiation (less than 0.01%). In the assay system used herein, NGF (100 ng per ml) mediated a cell cycle arrest as previously described (Chao[et al.], Cell, 68, 995-997 (1992)). In several experiments, in the presence of 10% fetal bovine serum NGF-mediated an induction of cell differentiation that observed in  $45 \pm 11\%$  of cells. When cells were subjected to NGF-mediated differentiation in the presence of 1% serum 5% to 10% of cells showed morphological changes. The differentiated cells showed typical neuron-like morphology. In parallel experiments, nine component peptides, colostrinin and colostrum were tested. The results are summarized in Table 1.